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REGULAR ARTICLE

Identification and functional analysis of two ZIP metal transporters of the hyperaccumulator *Thlaspi caerulescens*

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Abstract The heavy metal hyperaccumulator *Thlaspi caerulescens* expresses several *ZIP*-like genes at higher levels than their orthologues in non-hyperaccumulator species, but it is not clear why. To elucidate the function of the *T. caerulescens* orthologues of the *Arabidopsis thaliana ZIP5* and *ZIP6* genes, full-length cDNAs of *TcZNT5-LC* and *TcZNT6-LC* were cloned, their expression was examined and genes were expressed in *A. thaliana*. Transcript level analysis revealed the constitutively high expression of these two genes in *T. caerulescens* compared to *AtZIP5* and *AtZIP6* genes and differential expression of both genes when comparing two accessions of *T. caerulescens* with different metal accumulation properties. Expression of *TcZNT5-LC* in *A. thaliana* did not modify Cd or Zn tolerance, but mildly affected the root and shoot Zn concentrations towards a hyperaccumulator shoot to root concentration ratio. *A. thaliana zip5* knock-out mutants showed increased tolerance to Cd and decreased seed mineral concentrations. Expression of *TcZNT6-LC* enhanced the Cd sensitivity of *A. thaliana*, but no phenotype was observed for the *zip6* mutant. In conclusion, the changes in expression of *TcZNT5-LC* and *TcZNT6-LC* upon changes in Zn or Cd exposure indicate both genes act in metal homeostasis, but their CaMV 35S-mediated expression in *A. thaliana* does not create *T. caerulescens* hyperaccumulator phenotypes.

Keywords *TcZNT5* · *TcZNT6* · *Thlaspi caerulescens* · Hyperaccumulation · Metal transporter · Zinc · Cadmium

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Introduction

Thlaspi caerulescens, a Zn/Cd/Ni hyperaccumulator, has been used as a model species for dissection of the genetic and molecular mechanism of metal accumu-

lation and homeostasis (Assunção et al. 2003a). Hyperaccumulation of metals requires an adapted metal homeostasis allowing enhanced tolerance to metals and their accumulation. Therefore, functional and/or transcriptional modification of metal transporters, metal chelators and other proteins are needed to maintain a balance between plant metal uptake, distribution and storage (reviewed by Clemens 2001; Mäser et al. 2001; Cobbett and Goldsbrough 2002).

Members of the ZRT-IRT-like Protein (ZIP) family were the first metal transporters to be identified in plants (Eide et al. 1996). Fifteen *ZIP* genes have been identified in *A. thaliana* so far, based on whole genome sequencing. In general the ZIP transporter proteins have the capacity to transport a variety of divalent cations including Zn^{2+}, Fe^{2+}, Mn^{2+} and Cd^{2+} (Guerinot 2000) and the expression of *ZIP* genes is regulated by plant metal status due to environmental metal levels (Grotz et al. 1998). *AtIRT1* and *AtIRT2* have been described to have an essential role in maintenance of iron homeostasis (Connolly et al. 2002; Henriques et al. 2002; Varotto et al. 2002; Vert et al. 2001, 2002). *AtZIP1*, *AtZIP2* and *AtZIP3* confer Zn uptake activity when expressed in yeast (Grotz, et al. 1998). Genes encoding ZIPs have also been identified and characterized from other plant species, including soybean, *Medicago truncatula*, *Thlaspi caerulescens* and *T. japonica* (Assunção et al. 2001; Burleigh et al. 2003; Mizuno et al. 2005; Moreau et al. 2002; Pence et al. 2000; Plaza et al. 2007). *TcZNT1* and *TcZNT2*, the proposed *T. caerulescens* orthologues of the *A. thaliana AtZIP4* and *AtIRT3* genes respectively, are constitutively over-expressed in roots (Assunção et al. 2001; Pence et al. 2000). *TcZNT1* was shown to mediate high-affinity Zn uptake and low-affinity Cd uptake when expressed in yeast (Pence et al. 2000). Homologous genes *TjZNT1* and *TjZNT2* isolated from the Ni hyperaccumulator *T. japonica* were shown to be able to transport Zn, Cd and Mn (Mizuno et al. 2005).

Global transcriptomic studies revealed similar expression levels of *ZIP5* and *ZIP6* in roots of the Zn/Cd hyperaccumulator *A. halleri* and the non-hyperaccumulator *A. thaliana* (Weber et al. 2004). However, *ZIP6* in shoots was substantially higher expressed in *A. halleri* than in *A. thaliana* (Becher et al. 2004). Expression of *ZIP5* was up regulated in roots of *A. thaliana* upon Zn deficiency. Expression of the *T. caerulescens ZIP5* orthologue (*TcZNT5*) was higher compared with *A. thaliana* (van de Mortel et al. 2006). *TcZNT5* was previously cloned from *T. caerulescens* accession Prayon (Plaza et al. 2007). This gene was mainly expressed in roots with a much higher expression in Prayon than in the Ganges accession, which accumulates more Cd than the former (Plaza et al. 2007). These studies indicate that *ZIP5* and *ZIP6* and their orthologues are likely to have a role as possible Zn/Cd transporters in Zn homeostasis and Zn/Cd hyperaccumulation. Here we report the cloning of two new ZIP genes from *T. caerulescens* accession La Calamine (LC), which were named *TcZNT5-LC* and *TcZNT6-LC* based on their high similarity to the *A. thaliana* genes *AtZIP5* and *AtZIP6* respectively. Transcription levels of the two *T. caerulescens* genes were compared with their presumed *A. thaliana* orthologues and by comparing the *T. caerulescens* accessions LC and GA, which differ in Cd hyperaccumulation potential. Additional evidence for a functional role in Zn homeostasis was sought in studying T-DNA insertion knock-out mutants of *AtZIP5* and *AtZIP6* and in *A. thaliana* plants overexpressing *TcZNT5-LC* or *TcZNT6-LC* for a phenotypic change in their metal accumulation and their response to different Zn and/or Cd supply.

Materials and methods

Library screening

A cDNA library made from roots of *Thlaspi caerulescens* J. & C. Presl acc. La Calamine, as described by Assunção et al. (2001) was used for full-length cDNA cloning. Partial cDNA clones RR9nr066 and RR8nr089 in a pAD-GAL4-2.1 vector had been identified as putative orthologues of *AtZIP5* and *AtZIP6* (Rigola et al. 2006). cDNA fragments (approximately 500 bp for the *ZIP5* homologue and 650 bp for *ZIP6* homologue) cut from the pAD-GAL4-2.1 plasmid by *EcoR*I and *Xho*I were used as probes for cDNA library screening. The probes were labelled with [α-^{32}P]dATP using the Hexalabel™ DNA labelling kit (Fermentas, http://www.fermentas.com/). The isolated cDNA clones were sequenced by ABI PRISM BigDye terminator cycle sequencing technology v2.0, according to the manufacturer's instruction (Applied Biosystems; http://www.appliedbiosystems.com.), using an ABI3700 DNA analyzer. Sequence analysis was

performed using the standard BLAST method (http://www.ncbi.nlm.nih.gov/BLAST/). Transmembrane domains were defined according to TMHMM (http://www.cbs.dtu.dk/services/TMHMM/). Intracellular localization was predicted using PSORT (www.psort.nibb.ac.jp). Sequence alignment was conducted by MegAlign (DNAstar, Madison WI). Phylogenetic analysis was conducted using MEGA version 3.1 (Kumar et al. 2004)

DNA blot analysis

Genomic DNA was extracted from four *T. caerulescens* accessions: La Calamine (LC), Monte Prinzera (MP), Ganges (GA) and an accession from Hochobir, Austria, and four *Thlaspi* species: *T. japonicum*, *T. praecox*, *T. minimum* (three hyperaccumulators) and *T. perfoliatum* (a non-hyperaccumulator), using a modified CTAB method (Fulton et al. 1995). Digestion of genomic DNA, TAE gel electrophoresis, DNA blotting, prehybridization, hybridization and washing were all performed as described by Assunçaõ et al. (2001). Partial cDNA fragments digested from the pAD-GAL4-2.1 vector plasmids containing full-length cDNAs using *Eco*RI and *Xho*I for *TcZNT5-LC* (1,032 bp) and *TcZNT6-LC* (1,310 bp) were used as probes and labelled as described above.

Plant materials

Seeds of *A. thaliana* accession Columbia-0 (Col) and *T. caerulescens* accession LC were germinated on garden peat soil (Jongkind BV, The Netherlands). Three-week-old seedlings were transferred to hydroponics, three plants per pot filled with 1 L modified half-strength Hoagland nutrient solution (van de Mortel et al. 2006). After growth for 3 week on this solution, the *T. caerulescens* plants were transferred to the same nutrient solution with a deficient (0 μM), sufficient (100 μM), or high (1,000 μM) $ZnSO_4$ concentration on which they were grown for 7 additional days. The *A. thaliana* plants were transferred to the same nutrient solution with deficient (0 μM), sufficient (2 μM) or high (25 μM) $ZnSO_4$. The nutrient solution was renewed once a week during the first 3 week and thereafter twice a week. Germination and culture were performed in a climate chamber (20/15°C day/night temperature, 12 h (*A. thaliana*) or 14 h days (*T. caerulescens*).

For comparison of transcription levels of *TcZNT5* and *TcZNT6* between *T. caerulescens* accessions LC and GA, plants were grown as described above including two additional cadmium treatments (Zn 100 μM+Cd 1 μM and Zn 100 μM + Cd 10 μM).

Semi-quantitative RT-PCR

Shoots or roots of one pot containing three *A. thaliana* or three *T. caerulescens* plants per treatment were pooled and homogenized in liquid nitrogen. Total RNA of shoots or roots was extracted with Trizol (Invitrogen) following the manufacturer's instructions. Five micrograms of total RNA was used to synthesize cDNA with MLV reverse transcriptase (Invitrogen) and oligo (dT) as a primer (Invitrogen). The PCR amplification was performed with a 2-μl cDNA aliquot. The *AtZIP5* forward primer was 5′-ATGAGAATCACA CAAAACGTCAAGC-3′ and the reverse primer was 5′-TGGGATTCACCAGATTCCAC-3′; the *TcZNT5* forward primer was 5′-ACCGGAGCCGAGTTGTG-3′ and the reverse primer was 5′-TGGGCCATGATTT GAAGC-3′; the *AtZIP6* forward primer was 5′-GTCACCGGAACAGAGGCAGCAA-3 and the reverse primer was 5′-TTCACCGCAAGTCGTCAG CATCTT-3′; the *TcZNT6* forward primer was 5′-AGAGACGGAGACGCGGCGG-3′ and the reverse primer was 5′-CTGATGAAACGAAAGAGTAGCG-3′. Primer pairs for *Tubulin* were used as a control for using similar cDNA quantities for each sample. For *A. thaliana* the forward *Tubulin* primer was 5′-AAGCTTGCTGATAACTGTACTGGT-3′ and the reverse primer was 5′GGTTTGGAACTCAGTGA CATCA-3′; for *T. caerulescens* the forward primer was 5′-CTACGCACCAGTCATCTCT-3′ and the reverse primer was 5′-CGAGATCACCTCCTGGAACA-3′. For each comparative analysis several PCR cycles were run and the number of cycles which showed the best contrast between species, accessions or metal exposures was shown. For all for *Tubulin* amplifications 25 PCR cycles were performed for both *A. thaliana* and *T. caerulescens* samples. PCR fragments were separated on an ethidium bromide stained 1 % agarose TAE gel.

Construction of expression vectors

The full-length cDNAs of *TcZNT5-LC* and *TcZNT6-LC* were amplified from the pAD-GAL4-2.1 vector

plasmids by PCR. Gateway primers were used for PCR, the forward primer was 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTGATCGAATTA*GGATCC*TCTGC-3′, containing the attB1 sequence (underlined) and a *Bam*HI site (italic) in pAD-GAL4-2.1, and the reverse primer was 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTAATGGG*CTCGAG*AGTCGAC-3′, containing the attB2 sequence (underlined) and an *Xho*I site (italic) in pAD-GAL4-2.1. The PCRs were performed with the proofreading Pfu polymerase (Fermentas, http://www.fermentas.com) at 93°C for 5 min, followed by 30 cycles of 93°C for 1 min, 55°C for 1 min and 68°C for 3 min, and finished by an extension at 68°C for 10 min. PCR products were recombined into pDONR207 (Invitrogen, http://www.invitrogen.com) in a 10-μl BP Clonase (Invitrogen) reaction following the manufacturer's instruction. The fragments were transferred from their donor constructs to the binary over-expression vector pGD625 (de Folter et al. 2006), under the control of the double 35S CaMV enhancer, in a 10-μl LR Clonase (Invitrogen) reaction following the manufacturer's instruction. The binary constructs were introduced into *Agrobacterium tumefaciens* strain AGL0 by electroporation.

Plant transformation and metal tolerance/sensitivity growth assays

The binary constructs with full-length cDNAs of *TcZNT5-LC* and *TcZNT6-LC* were used to transform *A. thaliana* Col by the standard flower dip method (Clough and Bent 1998). The primary T1 transformants were selected on half-strength MS medium containing kanamycin (50 mg/ml). Kanamycin-resistant plants were transferred to soil, and the T2 seeds resulting from self-fertilization were collected. The T2 seeds were plated on the same selection medium and scored for kanamycin resistance. Transgenic lines that displayed a 3:1 segregation ratio for kanamycin resistance to sensitivity in the T2 generation indicating a single T-DNA insertion locus were selected for further analysis. In the T3 generation, we tested 12 T3 progeny plants from ten T2 plants each by PCR using the gene specific primers for *TcZNT5* and *TcZNT6* designed for the semi-quantitative RT-PCR. Genomic DNA was isolated using a modified CTAB method (Fulton et al. 1995). The lines for which all the 12 T3 progenies contained a T-DNA were deemed to be homozygous transgenics. T4 seeds from these plants were used for further experiments. The expression levels of *TcZNT5-LC* and *TcZNT6-LC* in the transgenic lines were determined by RT-PCR using total RNA extracted from T4 plants grown in soil for 30 days.

Selection of homozygous T-DNA insertion plants

SALK_009007 and SALK_116013 *A. thaliana* T-DNA insertion lines were obtained from the Nottingham Arabidopsis Stock Centre (NASC). SALK_009007 carries a T-DNA insert approximately 357 bp downstream of the start of the first exon of *AtZIP5* (At1g05300), while SALK_116013 carries a T-DNA insert approximately 363 bp downstream of the start of the first exon of *AtZIP6* (At2g30080) (Alonso et al. 2003). Genomic DNA was isolated from eight plants of SALK_009007 and 16 plants of SALK_116013. Homozygous mutants were identified by PCR using genomic DNA as template and separated combinations of primer LBb1, designed to fit the left border of the T-DNA insert (5′-GCGTGGACCGCTTGCTGCAACT-3′) (http://signal.salk.edu), and *AtZIP5* or *AtZIP6* specific primer pairs. The *AtZIP5* gene specific primers and the *AtZIP6* forward primer were the same as used in the semi-quantitative RT-PCR. The reverse primer for *AtZIP6* was 5′- TGCAACCACCAAGACCCAAA-3′. Seeds harvested from the identified homozygous T-DNA insertion plants and homozygous wild-type plants segregating in the respective SALK lines, were used for further phenotype screening.

Metal tolerance screening

Seeds were sterilized and sown on half-strength MS medium containing deficient or high Zn (0 μM or 200 μM), deficient Fe (0.5 μM), Cd (50 μM) and Fe deficient plus Cd (0.5 μM Fe+50 μM Cd). Half-strength MS medium was used as control. For each treatment, five replicates were used for each line with 20 seeds sown in each replicate. After being sown on media, seeds were kept at 4°C for 4 days in the dark to synchronize germination. Seedlings were grown in a 14 h light, 10 h dark cycle at 20°C day and 15°C night temperatures. The phenotypes were studied on 15-day-old plants by measuring root length.

Metal content measurement

Shoot metal concentrations were determined for wild-type *A. thaliana*, the overexpressing lines and T-DNA insertion mutant lines, with five replicates for each line. Plants were grown for 3 weeks in hydroponic solution (1/10 Hoagland's) containing 1 μM Zn, Cd and Ni. The solution was pH-buffered at 5.6 with 2 mM MES (2-morpholino-ethanesulphonic acid). Plants were rinsed with deionised water, separated into roots and shoots. Roots were then placed in an ice-cold solution of 1 mM $LaCl_3$ and 5 mM $CaCl_2$ for 20 min to desorb apoplastic metals. Roots and shoots were blotted dry with tissue paper and weighed. Roots and shoots were digested with 5 ml high purity $HNO_3/HClO_4$ (85:15, v:v). Metal concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS; Agilent ICP-MS 7500ce, Agilent Technologies, Santa Clara, CA, US).

The seed mineral concentrations were measured for wild type *A. thaliana* Col and *zip5* mutant plants. Seedlings were grown on a standard hydroponics solution suggested for *A. thaliana* (Tocquin et al. 2003), in a phytotron at 20°C with a relative humidity of 70% and a light intensity of 40 W/m^2 for 12 h/day. Seeds were harvested when completely mature and dry. Seed mineral concentrations were determined by Atomic Absorption Spectrometry (Perkin Elmer AAS 1100; Perkin Elmer, Rodgau-Judesheim, Germany) after acid digestion.

Statistical analysis

The phenotype data were analyzed by analysis of variance. The significance of the difference between mutant or over-expression lines with wild-type Col was determined by the use of least significant difference (LSD) at the 5% probability level.

Results

Cloning of full-length cDNA clones for TcZNT5-LC and TcZNT6-LC

Two partial *T. caerulescens* cDNA clones (RR9nr066 and RR8nr089), previously identified to have high DNA identity respectively to *AtZIP5* (At1g05300) and *AtZIP6* (At2g30080) (Rigola et al. 2006), were used as probes to screen a cDNA library prepared from roots of *T. caerulescens* accession La Calamine (LC) (Assunção et al. 2001). Three positive clones were obtained for each probe. One of the three positive clones detected with the RR9nr066 probe contained an open reading frame (ORF) of 1,068 bp covering the full predicted *AtZIP5* ORF and encoding a predicted protein of 355 amino acids and 37 kDa. It contains eight transmembrane domains (TMs) (Supplemental Fig. 1). The sequence has 86% DNA identity and 90% amino acid identity with *AtZIP5* and was named *TcZNT5-LC*. There is a putative metal binding domain containing a series of repeated histidine residues (HVHAHGHAHG) between TM4 and TM5, and it also has the conserved histidine residue in TM5 found in many members of the ZIP family. According to TAIR (www.arabidopsis.org), both AtZIP5 and the related protein AtZIP3 are targeted to the plasma membrane. Analysis by PSORT also predicted the TcZNT5-LC protein to be targeted to the plasma membrane. There are three other full-length cDNA sequences of *TcZNT5* from other accessions of *T. caerulescens*, one for GA and two for Prayon (PR), deposited at NCBI GenBank (AJ937738 (GA) and AJ937739 and AF292029 (PR)). The TcZNT5-LC and TcZNT5-PR amino acid sequences are identical, while the predicted protein encoded by *TcZNT5-GA* lacks three amino acids close to the N-terminus, compared to TcZNT5-LC and TcZNT5-PR.

The sequence of the longest of three positive clones identified by the RR8nr089 probe contains an ORF of 1,023 bp encoding a predicted protein of 340 amino acids, with a molecular weight of 35 kDa. The protein contains eight potential TMs (Supplemental Fig. 2) and is also predicted to be targeted to the plasma membrane by PSORT. The cDNA sequence has 86% DNA identity and 92% amino acid identity with *AtZIP6* and therefore the sequence was named *TcZNT6-LC*. The predicted TcZNT6-LC amino acid sequence contains the conserved histidine residue in TM4, but in contrast to most members of the ZIP family, TcZNT6-LC contains only two histidine residues as potential metal binding motifs in the variable region between TM3 and TM4.

The predicted amino acid sequences of *TcZNT5-LC* and *TcZNT6-LC* were aligned with other members of the ZIP gene family. Phylogenetic analysis (Fig. 1)

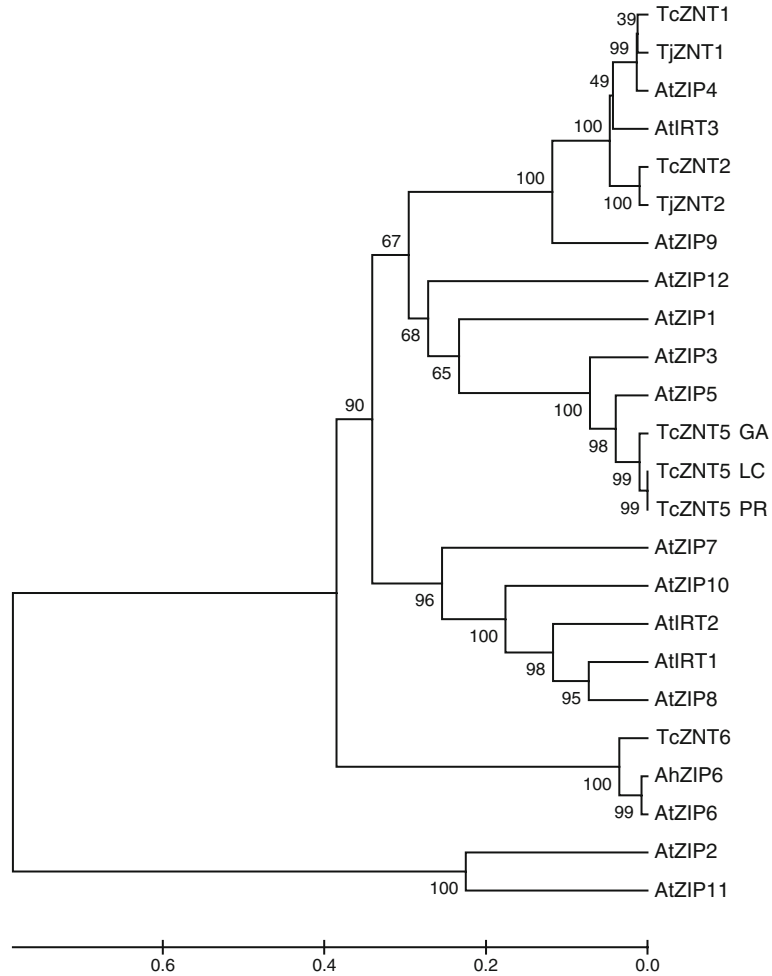

Fig. 1 Phylogenetic comparison of predicted protein sequences of TcZNT5 and TcZNT6 with those of 22 ZIP family members from *Arabidopsis thaliana*, *A. halleri*, *Thlaspi caerulescens* and *T. japonicum*. The phylogenetic analysis was conducted using MEGA version 3.1 (Kumar et al. 2004). GenBank accession numbers are: *AtIRT1* (NM_118089), *AtIRT2* (NM_001036593), *AtIRT3* (NM_104776), *AtZIP1* (NM_112111), *AtZIP2* (NM_125344), *AtZIP3* (NM_128786), *AtZIP4* (NM_100972), *AtZIP5* (NM_100409) *AtZIP6* (NM_128563), *AtZIP7* (NM_126440), *AtZIP8* (NM_148089), *AtZIP9* (NM_119456), *AtZIP10* (NM_102864), *AtZIP11* (NM_104468), *AtZIP12* (NM_125609), *AhZIP6* (AJ580315), *TcZNT1* (AF275751), *TcZNT2* (AF275752), *TcZNT5-PR* (AF292029), *TcZNT5-GA* (AJ937739), *TjZNT1* (AB206397), *TjZNT2* (AB175740). The *ruler* indicates genetic distance as the number of substitutions per unit time

revealed that TcZNT5-LC is closest to AtZIP5 as expected based on BLAST analysis, and both are closely related to AtZIP3. TcZNT6-LC is closest to AtZIP6 and both are more distantly related to the other ZIP family members. According to the previous studies on ZIP family members in *T. caerulescens* and the nomenclature used before (Pence et al. 2000; Assunção et al. 2001), we propose to maintain the provisional names for these two genes as *TcZNT5* and *TcZNT6*.

DNA blot analysis was performed to determine the copy number of the *TcZNT5* and *TcZNT6* genes in the *T. caerulescens* genome and in those of related *Thlaspi* species (Fig 2). We used restriction enzymes *Eco*RI (only for *TcZNT5*) and *Xba*I for genomic DNA digestion; *Eco*RI cuts once in the *TcZNT5-LC* cDNA sequence, *Xba*I does not cut in the *TcZNT5-LC* or *TcZNT6-LC* cDNA sequences. In all *T. caerulescens* accessions and other *Thlaspi* species tested, there were at least two bands for the *Eco*RI and *Xba*I digestions hybridized with the *TcZNT5-LC* probe (Fig. 2a), one strong band and one or two low intensity bands, suggesting addition-

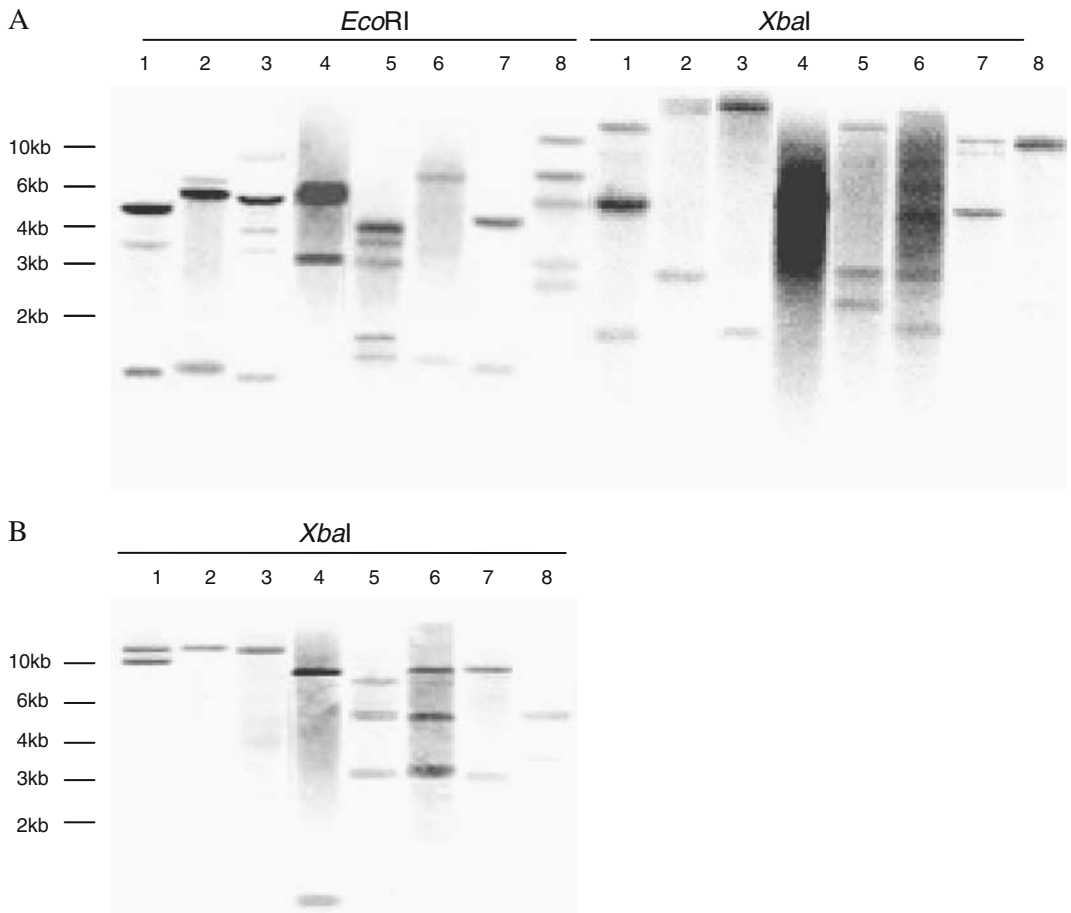

Fig. 2 DNA blot analysis of *ZNT5* and *ZNT6* in *T. caerulescens* accessions and *Thlaspi* species. Genomic DNA was digested with *Eco*RI or *Xba*I. DNA blots were hybridized with a *TcZNT5* (**a**) or *TcZNT6* (**b**) cDNA fragment probe. The numbers above the lanes designate different *T. caerulescens* accessions: La Calamine (1), Monte Prinzera (2), Ganges (3), Hochobir (7); or *Thlaspi* species: *T. japonica* (4), *T. praecox* (5), *T. minimum* (6) and *T. perfoliatum* (8). *Size markers* at shown on the *left*

al gene copies with lower similarity to the probe next to the *ZNT5* copies. As was previously found for *NRAMP* genes (Oomen et al. 2008), there is much stronger hybridization of the *TcZNT5-LC* probe to the lanes with *T. japonicum* genomic DNA than to other lanes. As the amount of genomic DNA loaded for *T. japonicum* was comparable to that of *T. caerulescens* (data not shown), it appears that multiplications of the *TjZNT5* gene have occurred in this species.

For *TcZNT6* some *T. caerulescens* accessions showed only one hybridizing fragment (Fig 2b), in accordance with the presence of only one copy of this gene in this species. *TjZNT6* is not multiplied to the same extent as *TjZNT5* in *T. japonicum*.

Expression of TcZNT5 and TcZNT6 in T. caerulescens

The role of *TcZNT5* and *TcZNT6* in metal accumulation and homeostasis in *T. caerulescens* (accession LC) was first evaluated by examining their expression upon exposure to various metal conditions. Transcript levels of *TcZNT5* and *TcZNT6* in shoots and roots were compared to those of their *AtZIP5* and *AtZIP6* orthologues in *A. thaliana* using semi-quantitative RT-PCR on 4-week-old, hydroponically grown plants. In general, expression of *TcZNT5* and *TcZNT6* is higher than that of *AtZIP5* and *AtZIP6*, both in roots and shoots (Fig. 3). Expression of *AtZIP5* was induced under Zn deficiency, whereas expression of

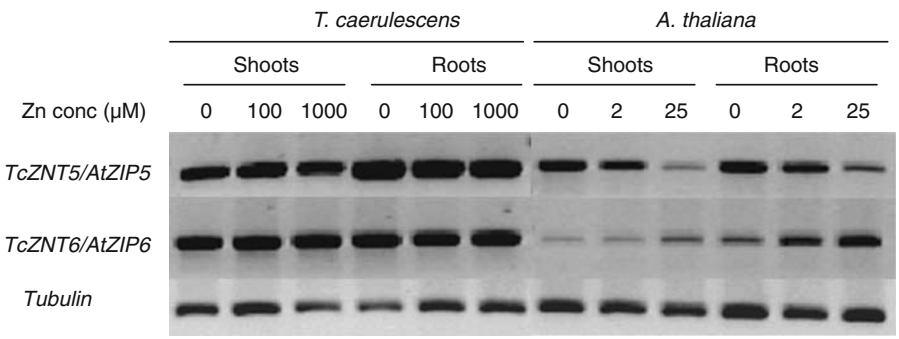

Fig. 3 Semi-quantitative RT-PCR analysis of *T. caerulescens* *TcZNT5* and *TcZNT6* and their *A. thaliana* orthologues *AtZIP5* and *AtZIP6*. Shoots and roots were used from plants grown at 0, 100 and 1,000 μM Zn for *T. caerulescens* and 0, 2 and 25 μM Zn for *A. thaliana*. RT-PCR was performed using specific primer pairs for each gene. RT-PCR (30 cycles) was used to amplify a 424-bp fragment for *TcZNT5*, a 605-bp fragment for *TcZNT6*, a 593-bp fragment for *AtZIP5* and a 679-bp fragment for *AtZIP6*. *Tubulin* amplification (25 cycles) was used as control for equal use of cDNA

AtZIP6 was induced by high Zn supply. The transcript level of *TcZNT5* was higher in roots than in shoots, while *TcZNT6* was expressed at a similar level in shoots and roots under the Zn levels tested. In *A. thaliana*, *AtZIP5* is expressed at a similar level in roots and shoots, while *AtZIP6* expression is higher in roots than in shoots.

As *T. caerulescens* is a Zn/Cd hyperaccumulator, we further tested the changes of transcript levels of *TcZNT5* and *TcZNT6* in roots and shoots upon exposure to deficient (0 μM), sufficient (10 μM and 100 μM) and high Zn (1,000 μM) conditions or with sufficient Zn (100 μM) in the presence of low Cd (1 μM) or high Cd (10 μM) concentrations in two *T. caerulescens* accessions, LC and GA. These two accessions are similar in Zn/Cd tolerance, but under the conditions we used, GA accumulates more Zn and Cd than LC (Assunção et al. 2003b). As shown before, *TcZNT5* was predominantly expressed in roots in both accessions (Fig. 4), but with a higher expression in LC compared to GA. Although much less than what we observed for *AtZIP5*, expression of *TcZNT5* was also slightly up-regulated in roots under Zn deficiency in both accessions. Cd treatment did not alter the expression level of *TcZNT5* in LC or GA roots or leaves. There was a difference in expression of *TcZNT6* between two accessions in roots. *TcZNT6* was expressed at similar levels in shoots and roots in GA, irrespective of the metal supply. In LC the expression in shoots was the same, but in roots expression was lower than in GA and down-regulated in response to 10 μM Cd (+100 μM Zn).

Phenotypic analysis of Arabidopsis zip5 T-DNA insertion mutants and TcZNT5-LC expressing plants

Investigating the function of *TcZNT5* and *TcZNT6* in *T. caerulescens* is cumbersome in the absence of a collection of T-DNA insertion mutants like in *A. thaliana*. Therefore we analyzed T-DNA insertion mutants of their orthologues, *AtZIP5* and *AtZIP6*, in *A. thaliana*. Homozygous *zip5* T-DNA insertion plants and wild-type (WT) sister plants were isolated by PCR screening (data not shown). RT-PCR confirmed the expression of *ZIP5* in the WT plants. No expression was detected in the mutant (data not shown) indicating that the T-DNA is not spliced from the transcript and that the insertion is likely to cause a loss-of-function null allele.

In addition the *TcZNT5-LC* full-length cDNA was expressed under control of the constitutive CaMV 35S promoter in *A. thaliana* Col. Three independent homozygous single-locus transgenic expression lines were obtained. The *TcZNT5-LC* transgene expression was higher in line no. 6 than in the other two lines, as found by semi-quantitative RT-PCR (Fig. 5).

When grown in soil, both *zip5* mutants and transgenic plants expressing *TcZNT5-LC* showed no visible morphological differences compared to WT plants. When screened for root growth on vertical plates, *zip5* seedlings had 19.3% longer roots than WT seedlings when grown on medium containing 50 μM Cd ($P<0.05$), suggesting increased Cd tolerance. However this difference disappeared when plants were grown on medium with low Fe supply

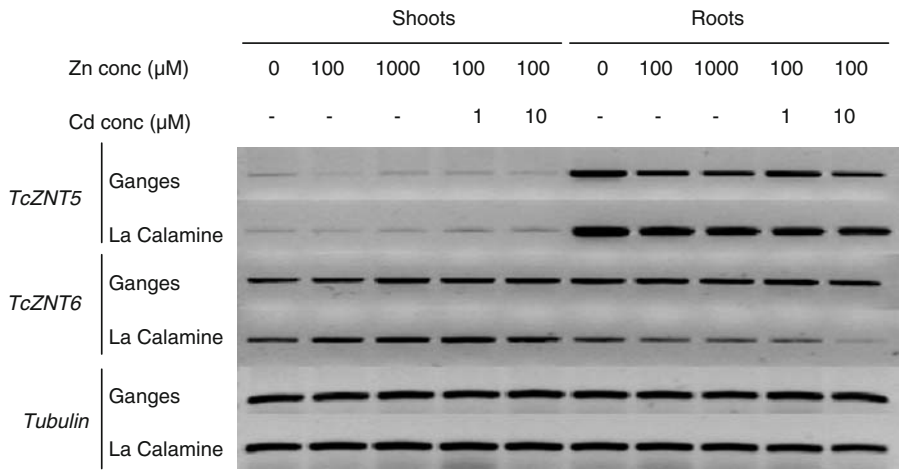

Fig. 4 Zn and Cd exposure effects on *TcZNT5* and *TcZNT6* transcript levels in *T. caerulescens* accessions Ganges and La Calamine. Shoots and roots were used from plants grown at 0, 100 and 1,000 μM Zn, or 100 μM Zn supplemented with 1 or 10 μM Cd. RT-PCR was performed (25 cycles) using gene specific primers to amplify a 424-bp fragment from *TcZNT5* and 605-bp fragment from *TcZNT6*. *Tubulin* amplification (25 cycles) was used as a control of equal cDNA use

(0.5 μM) in the presence of Cd (Fig. 6a). In contrast, no significant differences in root lengths were detected for *TcZNT5-LC* expressing lines compared with WT at low Zn, low Fe and high Cd supply (Fig. 6b).

Mineral concentrations were determined in roots, shoots and seeds of the *zip5* mutant plants and roots and shoots of *A. thaliana* lines expressing *TcZNT5-LC* grown in hydroponics. Shoot Fe and Zn concentrations were significantly higher in *zip5* (27 % and 18 %, respectively), while root Cu and Cd concentrations were significantly lower (55% and 26%, respectively) compared with WT ($P<0.05$) (Fig. 6c). Seed Fe, K, Mg and P concentrations were significantly decreased in *zip5* (Fig. 6d). An evaluation of growth related traits also showed that fruit length and seed weight were a little, but significantly, decreased in *zip5* (Fig. 6e), indicating that inactivation of *ZIP5* disturbs the normal seed setting of *A. thaliana* plants. Plants expressing *TcZNT5-LC* did not show a univocally strong change in mineral accumulation. However, two of the three transgenic lines (no. 4 and no. 6) showed a significant decrease in root Zn concentrations, 40% and 78% of WT (Fig. 6f), and the line with higher expression of *TcZNT5-LC* (no. 6) also showed a significantly enhanced shoot Zn concentration (50% higher) when compared to wild type ($P<0.05$) (Fig. 6g). Consequently their shoot to root Zn concentration ratios increased, more resembling *T. caerulescens* ratios. The other transgenic line (no. 2) showed a significant increase in the root Cd concentration ($P<0.05$) (Fig. 6f).

Phenotypic analysis of Arabidopsis zip6 T-DNA insertion mutants and TcZNT6-LC expressing plants

A. thaliana T-DNA insertion mutants of *AtZIP6* and transgenic lines expressing *TcZNT6-LC* were analyzed

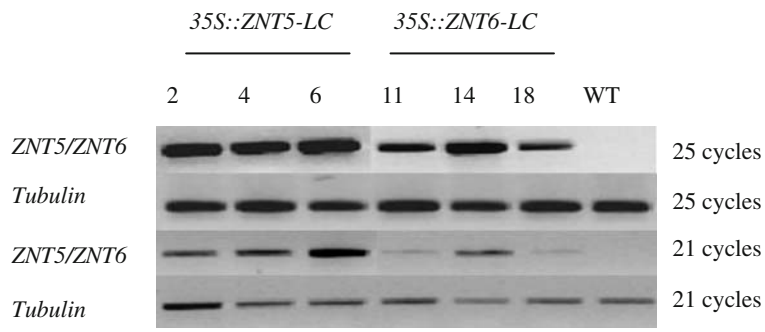

Fig. 5 Semi-quantitative RT-PCR analysis of *TcZNT5-LC* or *TcZNT6-LC* expression of wild-type (WT) and homozygous transgenic *A. thaliana* lines transformed with a CaMV 35S::*TcZNT5-LC* (*ZNT5*) or CaMV 35S::*TcZNT6-LC* (*ZNT6*) construct. *Tubulin* amplification was used as a control of equal cDNA use. The number of PCR cycles is indicated

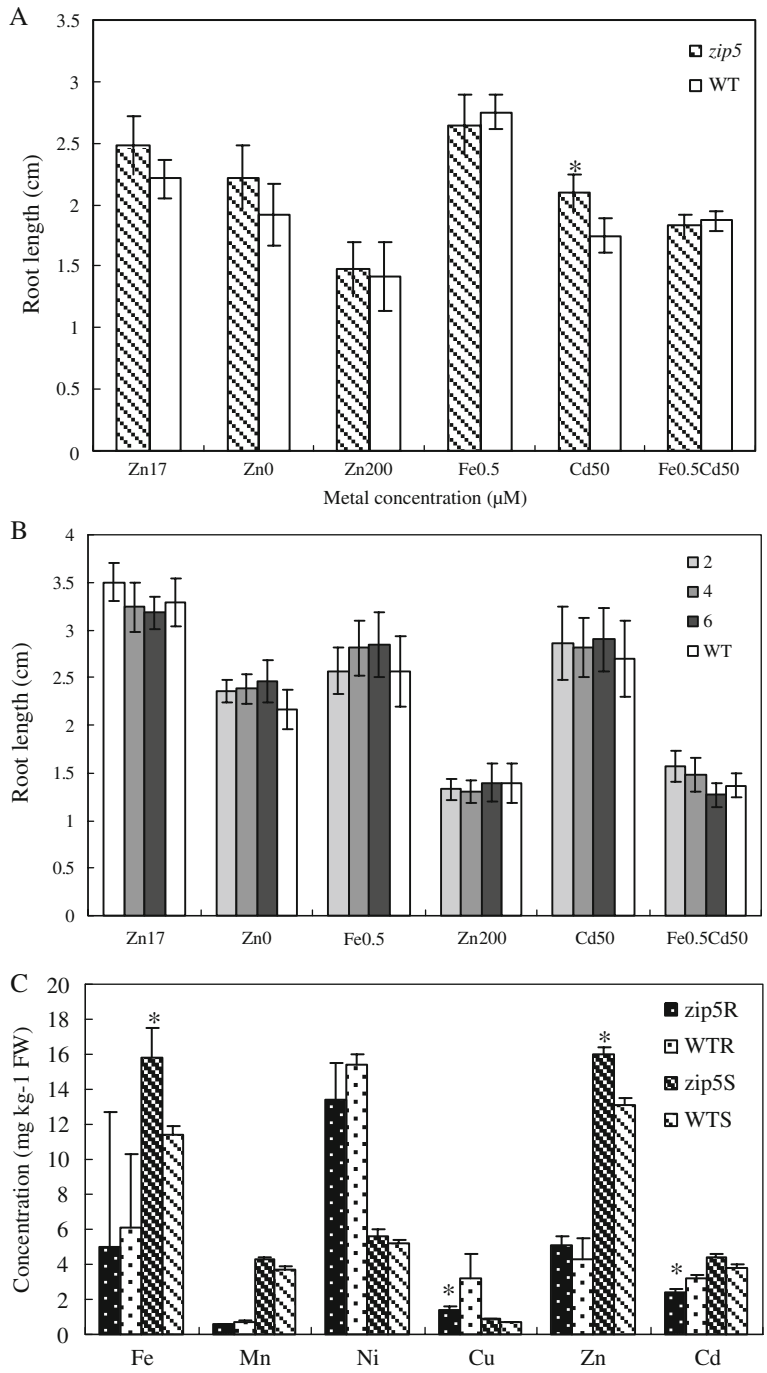
A
Root length (cm)
zip5
WT
Zn17
Zn0
Zn200
Fe0.5
Cd50
Fe0.5Cd50
Metal concentration (μM)
B
Root length (cm)
2
4
6
WT
Zn17
Zn0
Fe0.5
Zn200
Cd50
Fe0.5Cd50
C
Concentration (mg kg-1 FW)
zip5R
WTR
zip5S
WTS
Fe
Mn
Ni
Cu
Zn
Cd

Fig. 6 *A. thaliana zip5* T-DNA insertion loss-of-function mutants and transgenic lines expressing *TcZNT5-LC*, showed phenotypic changes in tolerance and mineral accumulation. Average root length (± SE) of 15-day-old *A. thaliana* wild-type (WT) and *zip5* seedlings (**a**) or *35S::TcZNT5-LC* expression lines nos. 2, 4 and 6 (**b**), germinated and grown on half-strength MS medium containing different concentrations of Zn, Fe and Cd. Five replicates with each 12 to 20 seedlings were measured for each treatment. **c** Mineral concentrations (± SE) in *zip5* mutant roots (zip5R) and shoots (zip5S) or in wild-type roots (WTR) and shoots (WTR) of hydroponically grown plants (1/10 Hoagland's solution) supplied with 100 μM Fe-EDTA and 1 μM Zn, Cd and Ni. **d** Seed Zn, Mn, Fe, K and Ca concentrations (± SE) of wild-type (WT) and *zip5* mutant plants. **e** Average number of seeds per fruit (SNF), fruit length (FL, in cm) and seed weight (SW, in mg per 100 seeds) of WT and *zip5* mutant plants. (**f**) and (**g**) Mineral concentrations (± SE) in roots (**f**) and shoots (**g**) of hydroponically grown (1/10 Hoagland's solution containing 100 μM Fe-EDTA and 1 μM Zn, Cd and Ni) 14-day-old seedlings of *35S::TcZNT5-LC* expression lines nos. 2, 4 and 6. For (**c**), (**f**) and (**g**) we divided the actual root Fe concentrations by 100 and the actual root Zn and root and shoot Cd concentrations by 10 in order to show all mineral concentrations relative to each other in the same graphs. * Significantly different at $P<0.05$. Significance is determined by one-way ANOVA. Five replicates were measured for each treatment in (**c**), (**f**) and (**g**)

for their phenotype differences compared with WT. Three independent homozygous single-locus transgenic expression lines were studied. One of the three lines (no. 14) showed a high expression level of the transgene, the other two lines showed lower expression of the transgene, but still higher than expected for the *AtZIP6* gene (Fig. 5).

Like *zip5* mutants and *TcZNT5-LC* expressors, *zip6* mutants and plants expressing *TcZNT6-LC* did not show obvious morphological alterations compared to WT when they were grown in soil. When screened for root growth on vertical plates, no significant differences in root length were detected when comparing *zip6* mutants with WT plants, irrespective of the metal exposures (Fig. 7a). However, for transgenic lines, high expression of *TcZNT6-LC* (no. 14) resulted in significantly reduced root length compared to the WT plants and plants with modest *TcZNT6-LC* expression (no. 11 and no. 18) on medium supplemented with Cd (50 μM) ($P<0.001$), both under sufficient and deficient Fe supply, thus enhancing the Cd sensitivity (Fig. 7b). The exposures to Zn deficiency or excess Zn showed no significant differences in root length.

Mineral concentrations were determined in roots and shoots of *zip6* mutant plants and *TcZNT6-LC* expressing plants grown in hydroponics. The root Fe concentration was significantly lower (17%) in *zip6* plants than in WT plants, but the shoot Mn concentration was higher (25%) in *zip6* than in WT (Fig. 7c) ($P<0.05$). The three independent *TcZNT6-LC* expressing lines all showed significantly higher shoot Mn concentrations compared with WT ($P<0.05$) (Fig. 7d). Only line 11 showed a significantly higher Fe concentration in roots ($P<0.05$) (Fig. 7e).

Discussion

Screening of a *T. caerulescens* root cDNA library identified full-length cDNA clones of two ZIP family members, *TcZNT5-LC* and *TcZNT6-LC*, the latter of which had not been identified in *T. caerulescens* before. The predicted proteins encoded by the two genes share all common features found in ZIP proteins: they have eight putative transmembrane domains (TM); they contain a histidine repeat in the variable region, which has been proposed as the metal binding and/or sensing site (Grossoehme et al. 2006); and they have a conserved histidine residue in TM4 or TM5, which is predicted to occupy the polar face of the amphipathic helix and has a role in substrate transport through the membrane (Eng et al. 1998; Guerinot 2000).

The N-termini of the predicted protein sequences of the four identified *TcZNT5* alleles from accessions LC, GA and PR (two alleles) are different from the predicted protein sequence of the *A. thaliana AtZIP5* gene. All *T. caerulescens* alleles appear to lack one of the codons encoding a phenylalanine residue. However, when comparing the *AtZIP5* predicted protein sequence to that of the related gene *AtZIP3*, it shows that AtZIP3 is also lacking the phenylalanine residue and an adjacent leucine residue, suggesting that the phenylalanine residue may not be critical in terms of providing proper protein function. Also among the four *T. caerulescens* alleles there are some differences. Most striking is the absence of three codons encoding Lys8-Leu9-Leu10 in the predicted TcZNT5-GA protein sequence. It will be interesting to determine if this difference affects the subcellular localization of the TcZNT5 protein.

Both *TcZNT5* and *TcZNT6* are more or less constitutively expressed in *T. caerulescens* La Calamine, with both root and shoot expression staying fairly constant over a wide range of Zn supplies

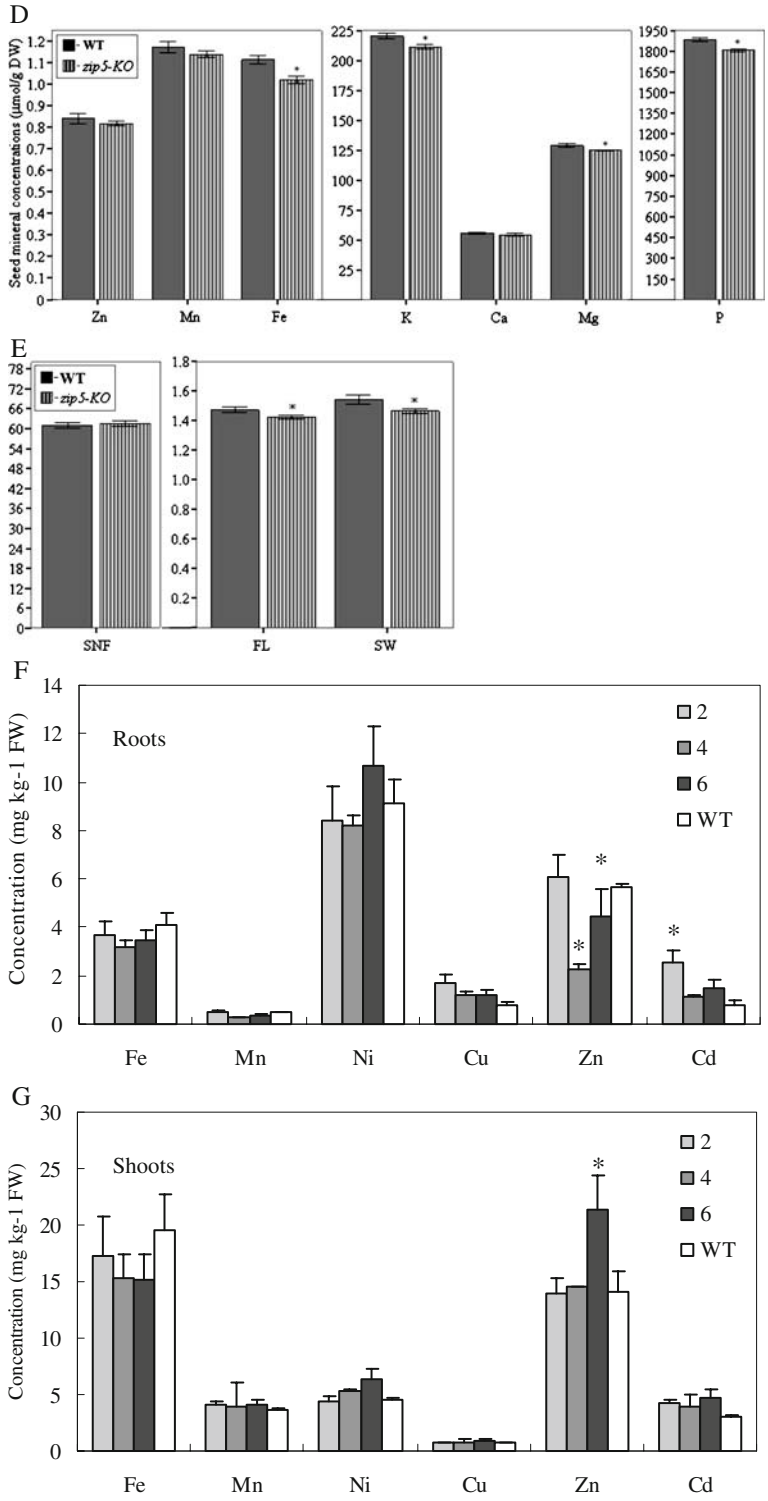

Fig. 6 (continued)

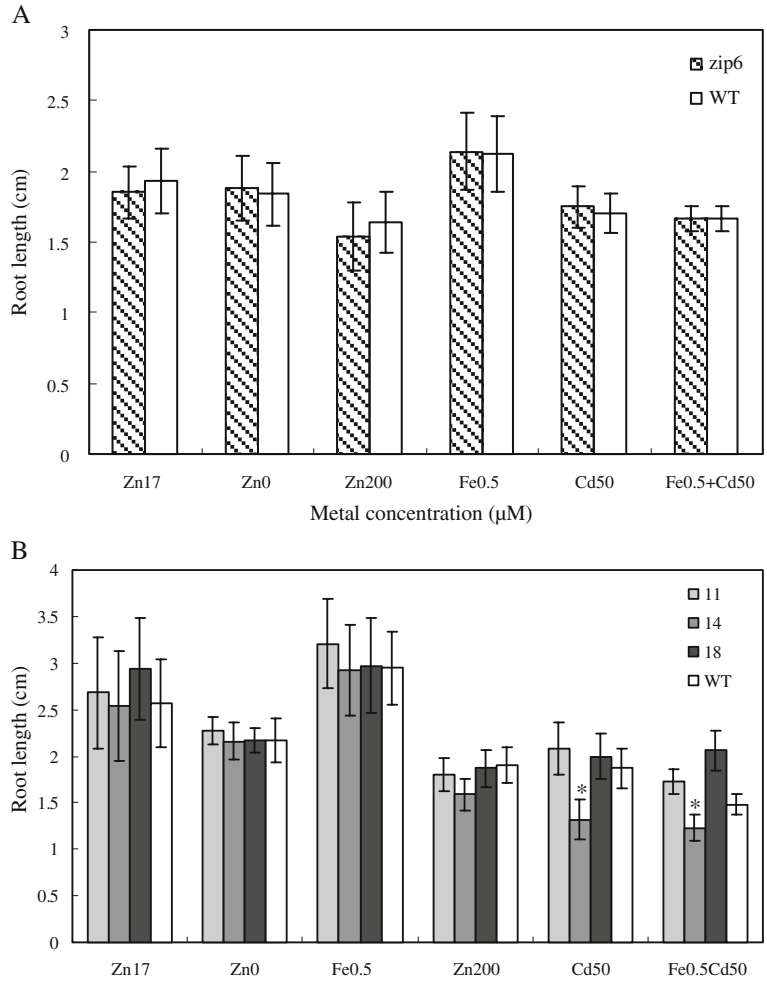

Fig. 7 *zip6* T-DNA insertion loss-of-function mutants and transgenic *A. thaliana* plants expressing *TcZNT6-LC* showed phenotypic changes in tolerance and mineral accumulation. Average root length (± SE) of 15-day-old *A. thaliana* wild-type (WT) and *zip6* seedlings (**a**) or *35S::TcZNT6-LC* expression lines nos. 11, 14 and 18 (**b**), germinated and grown on half-strength MS medium containing different concentrations of Zn, Fe and Cd. Five replicates with each 12 to 20 seedlings were measured for each treatment. * indicates significantly different at $P<0.001$ as determined by one-way ANOVA. Five replicates, each with 12 to 20 seedlings, were measured for each treatment. **c** Mineral concentrations (± SE) in *zip6* mutant roots (zip6R) and shoots (zip6S) or in wild-type roots (WTR) and shoots (WTR) of hydroponically grown plants (1/10 Hoagland's solution) supplied with 100 μM Fe-EDTA and 1 μM Zn, Cd and Ni. **d** and **e** Mineral concentrations (± SE) in roots (**d**) and shoots (**e**) of hydroponically grown (1/10 Hoagland's solution containing 100 μM Fe-EDTA and 1 μM Zn, Cd and Ni) 14-day-old seedlings of *35S::TcZNT6-LC* expression lines nos. 11, 14 and 18. For **c**, **d** and **e** we divided the actual root Fe concentrations by 100 and the actual root Zn and root and shoot Cd concentrations by 10 in order to show all mineral concentrations relative to each other in the same graphs. * Significantly different at $P<0.05$. Significance is determined by one-way ANOVA. Five replicates were measured for each treatment in (**c**), (**d**) and (**e**)

(0–1,000 μM). This is different from the Zn-status-dependent regulation of the expression of their orthologues *AtZIP5* and *AtZIP6* in *A. thaliana* (Fig 3). Both *TcZNT5* and *TcZNT6* are also expressed at a higher level in *T. caerulescens* than their orthologues in *A. thaliana*. The RT-PCR results presented here confirm the root micro-array expression data for *TcZNT5* and *AtZIP5* as determined by van de Mortel et al. (2006). They did not report a higher root expression of *TcZNT6* compared to *AtZIP6* as the difference did not exceed the 5-fold threshold level used as a cut-off in that analysis. A similarly high and constitutive expression was previously reported for the *TcZNT1* and *TcZNT2* genes in

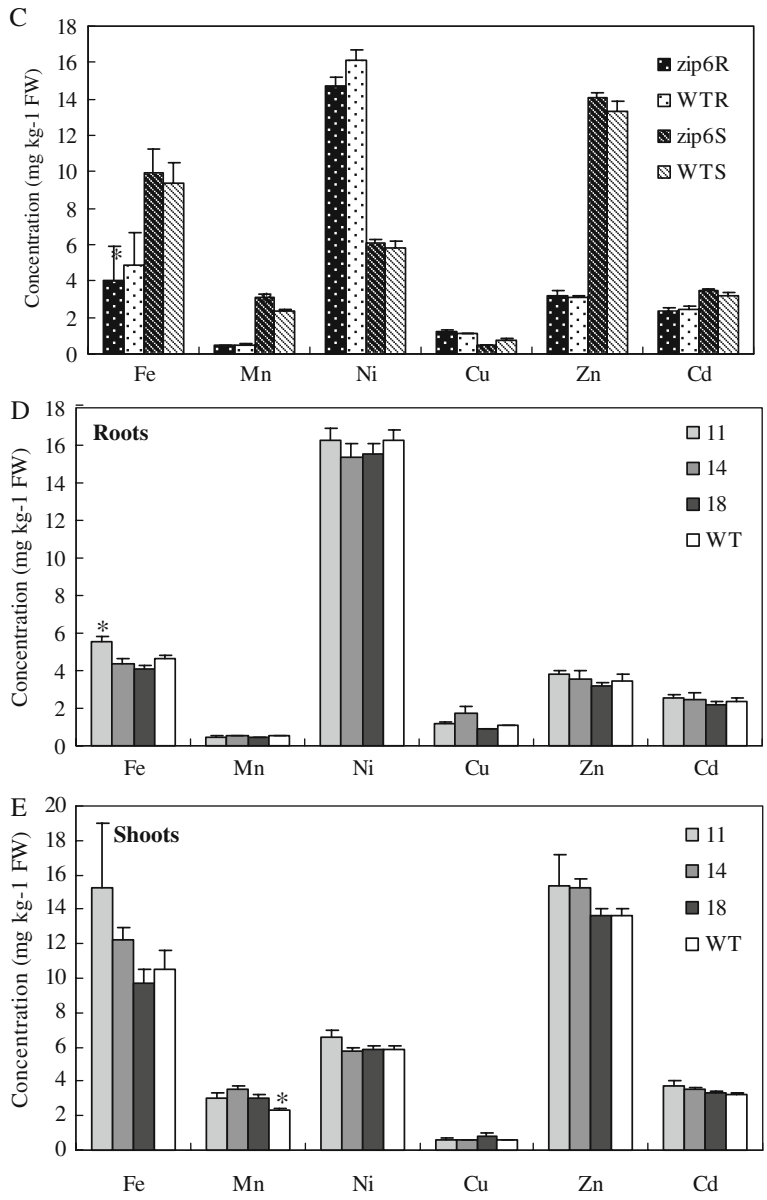

Fig. 7 (continued)

T. caerulescens and the *TgMTP1* and *AhMTP1* genes in the metal hyperaccumulator species *T. goesingense* and *A. halleri* (Assunção et al. 2001; Dräger et al. 2004; Pence et al. 2000; Persans et al. 2001). As Zn hyperaccumulation in *T. caerulescens* is a constitutive trait involving strongly enhanced metal uptake and root to shoot translocation (Assunção et al. 2003a), higher cation transport capacity will be needed for taking up metals from soil and for intracellular storage and allocation (Lasat et al. 1996). *T. caerulescens* seems to have dealt with the demand for higher transport capacity by increasing the expression of the available zinc transporter genes.

The similarities in protein sequence between *TcZNT5* in *T. caerulescens* and *AtZIP5* in *A. thaliana* and the predominant expression of *TcZNT5* in roots provide evidence for a presumed function of *TcZNT5* in cellular Zn uptake mainly in roots of *T. caerulescens*. Since *AtZIP5* is expressed more or less equally in roots and shoots, it is likely to have such a function

in both roots and shoots. Nevertheless, the *A. thaliana* knock-out *zip5* mutant did not show a strong phenotype when exposed to different metal supplies, which suggests that the *AtZIP5* gene function is largely redundant. This is not unexpected in view of the presence of the highly similar *AtZIP3* gene. Double mutant *zip3zip5* plants need to be studied to examine this further.

When comparing the two accessions of *T. caerulescens*, expression of *TcZNT5* in roots is slightly higher in LC than in GA with or without Cd exposure. Plaza et al. (2007) also reported a higher expression of *TcZNT5* in GA than in PR, an accession very similar to LC both in origin and metal accumulation characteristics (Lombi et al. 2001) and in *TcZNT5* allele coding sequence. As GA is a much better Cd hyperaccumulator than LC under the investigated conditions (Assunção et al. 2003b), *TcZNT5* is unlikely to play a dominant role in Cd uptake in *T. caerulescens* roots. Since studying the gene functions in *T. caerulescens* by transgenics is cumbersome, we aimed to mimic the higher expression in GA by expression of *TcZNT5-LC* under the control of the CaMV 35S promoter in *A. thaliana*. The high expression of *TcZNT5-LC* in *A. thaliana* also promoted a shift in accumulation of Zn towards leaves, although clearly not at the level of a Zn hyperaccumulator and only in two of the three expressors. Obviously only altering expression of one gene, *TcZNT5*, is not enough to fully mimic a hyperaccumulation phenotype and it may well be that tissue-specific expression is needed rather than the general, high ectopic expression created by the CaMV 35S promoter.

Comparison of the *TcZNT6* expression in GA and LC showed a lower expression in LC roots at equal Zn supply and a clear down-regulation in LC upon increased Cd supply. Knowing that GA is a slightly better Zn hyperaccumulator and a much better Cd hyperaccumulator than LC under the conditions tested (Assunção et al. 2003b), this supports a role for this protein in shoot Zn storage and suggests some ability to transport Cd. Similar to the expression pattern of *TcZNT6* in *T. caerulescens* LC, *AhZIP6* is also more highly expressed in shoots than in roots of the Zn/Cd hyperaccumulator *A. halleri* (Becher et al. 2004). The main difference between *A. thaliana* and its hyperaccumulating relative is a higher accumulation of metals in the shoots. Therefore the stronger expression of *TcZNT6/AhZIP6* in shoots and the increased expression of *AtZIP6* upon increasing Zn conditions further suggest these genes may play a role in storage or detoxification of Zn, or in maintaining homeostasis of other metals like Fe or Mn, which is affected by enhanced Zn or Cd exposure, as was observed by van de Mortel et al. (2006; 2008).

In conclusion, *TcZNT5* and *TcZNT6,* two ZIP genes cloned from *T. caerulescens*, are constitutively highly expressed and likely to encode metal transporters with Zn, Cd, and Fe or Mn transport ability. Additional studies are needed to further elucidate their exact role in plant mineral homeostasis and metal hyperaccumulation.

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